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ORIGINAL PAPER

A comparative study of *Epipactis atrorubens* in two different forest communities of the Middle Urals, Russia

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Abstract The objective of this study was to compare eco-physiological and morphological parameters of a regionally endangered orchid species, *Epipactis atrorubens* (Hoffm. ex Bernh.) Bess., growing in two forest communities (on serpentine and granite outcrops) of the Middle Urals, Russia. Biodiversity, dominance, and phytocoenosis studies showed the colonization of a wide range of plant species on both sites. The physicochemical properties of the soil, chemical composition and morphological features of *E. atrorubens,* growing under technogenic conditions (asbestos deposits), on serpentine outcrops and in the natural environment of the granite massif were studied for the first time. The serpentine substrate differed from the granite one by its greater stoniness, circumneutral pH and lower contents of available nitrogen and phosphorus. Extremely high concentrations of magnesium were found in the serpentine soil, some 79 times higher than in the granite substrate. High concentrations of nickel (94 times), chromium (59 times), cobalt (17 times), and iron (4 times) were found in the serpentine substrate, higher than in the granite substrate. The differences between the sites for available metal contents and for root and shoot metal contents were significantly less. Concentrations of most of the metals in the roots were higher than in the shoots. Despite higher metal concentrations and lower nitrogen and phosphorus levels in serpentine soils, *E. atrorubens* had a larger population and greater viability compared to those growing on granite. Plants on serpentine outcrops were characterized by the formation of a larger number of fruits, greater root lengths and thicker leaf blades, compared to plants on granites. The well-developed orchid mycorrhizae contributed to the survival of this species under unfavorable serpentine conditions. Hence, serpentine outcrops formed due to the mining of asbestos could be a suitable substrate for the light-demanding *E. atrorubens* due to its capacity to adapt to dry, rocky, nutrient-depleted soils and limited competition from other plants.

Keywords Orchids · Forest phytocoenosis · Serpentine · Granite · Metal accumulation

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Introduction

The conservation of biodiversity is a current scientific and practical challenge. Anthropogenic activities lead to significant changes in vegetation cover and a reduction in the natural habitats of rare and endangered species, which results in a decrease in the number of individuals, and sometimes complete extinction (Jurkiewicz et al. 2001; Vakhrameeva et al. 2008; Swarts and Dixon 2009). To date, information has accumulated on the settlement of rare species, including some orchids, in man-made habitats (Jurkiewicz et al. 2001; Adamowski 2006; Rewicz et al. 2017). A comprehensive study of rare and endangered Orchidaceae genera and species, their biological and ecological features, the structure and dynamics of populations, and their association with other components of the community allows for the prediction of their development and improvement of protective measures (Tsiftsis et al. 2008; Vakhrameeva et al. 2008; Swarts and Dixon 2009).

An endangered orchid species of forest plant communities of the Middle Urals is *E. atrorubens* (Hoffm. ex Bernh.) Bess. (Mamaev et al. 2004; Red Book of Sverdlovsk Region 2008), which is also listed in the European Red List of Vascular Plants under the category of "Least Concern" (Bilz et al. 2011). This species is widely distributed in boreal, temperate and submeridional zones, mostly in the north to the subarctic, in the south to the Mediterranean, and in the east to Western Siberia and the Caucasus, excluding Eastern Siberia and the Far East (Buttler 1991; Delforge 2006). This species is found in deciduous, coniferous and mixed forests, along forest edges, and in shrub thickets with sunlight $> 10\%$ (Vakhrameeva et al. 2008). It prefers neutral or alkaline, dry or medium dry, well-aerated soils (Landolt 1977; Ellenberg et al. 1991). *Epipactis atrorubens* is a good colonizer on stony industrial deposits of talc, limestone, gypsum, zinc and mine tailings (Jurkiewicz et al. 2001; Adamowski 2006; Shefferson et al. 2008). However, low genetic variation can hinder the ability of this species to adapt to a new environment (Hens et al. 2017).

According to Delforge (2006) and Djordjević et al. (2016), *E. atrorubens* can also grow on serpentine areas. One such population was found recently in the Middle Urals on serpentine outcrops of asbestos deposits (Filimonova et al. 2019).

Serpentine soils are generally unfavorable for plant growth due to their poor physical and chemical properties (Kristy et al. 2005; Rajakaruna and Boyd 2014; Kumar et al. 2017). They have low nitrogen, phosphorus, potassium, and calcium contents, high levels of magnesium and iron, relatively high concentrations of nickel, chromium, cobalt, and pH from neutral to alkaline—which altogether, have detrimental and toxic effects on plants (Brady et al. 2005; Van der Ent et al. 2015; Kumar et al. 2017). Although numerous studies have been carried out on plants of serpentine areas (Kristy et al. 2005; Kazakou et al. 2010; Rajakaruna and Boyd 2014; Djordjević et al. 2016), adaptations of *E. atrorubens*, abundantly growing on serpentine outcrops, have been less studied. Therefore, a study of *E. atrorubens* colonizing serpentine deposits compared with the species from another forest community (granite outcrops), may make it possible to identify specific adaptations allowing this species to survive under stressful conditions. This will contribute to the development of measures for forest resource conservation.

This research aimed to: (1) analyze phytocoenotic conditions and physicochemical soil parameters of two forest plant communities of the Middle Urals (serpentine and granite outcrops); (2) compare the chemical composition (nitrogen, phosphorous and metal concentrations) of *E. atrorubens* colonizing these sites; and, (3) investigate the morphological features of this species and the degree of mycorrhizal association.

Materials and methods

Study site description

The research was carried out in the territory of the Middle Urals (a subzone of the southern taiga). Two naturally colonized populations of *E. atrorubens* were studied: P-1 on serpentine outcrops of the Shilovsky deposit (57°44′55″N, 60°12′38″E; altitude: 340 m); P-2 on the summit of Motaikha Mount (56°57′44″N, 60°19′46″E; altitude: 439 m), Fig. 1.

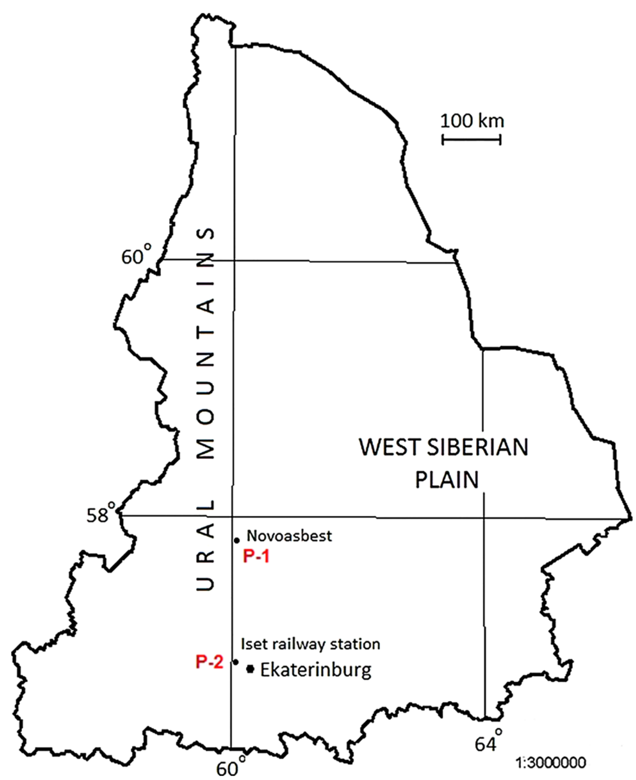

Fig. 1 A schematic map of the Middle Urals (Russia) with collection localities: P-1, population of *E. atrorubens* growing on serpentine outcrops of the Shilovsky deposit; P-2, population of *E. atrorubens* growing on granite outcrops of Motaikha Mount

P-1 is located 132 km north of Ekaterinburg city, 2.5 km from Novoasbest village within the serpentine massif (Fig. 2a, b). The area is confined to lenticular deposits of talc-chlorite-carbonate rocks. Open cast mining operated from 1952 to 1992 for the extraction of fibrous asbestos. The average content of asbestos in the rocks was 4–5%. After the extraction of asbestos fibers, the waste materials were deposited along road sides, resulting in the formation of 2–3 tiered serpentine deposits. The height of the deposits is 30–35 m in an area of approximately 40 ha.

P-2 was found 50 km north-west of Ekaterinburg city in a forest plant community (Fig. 2c, d). The soils of this granite outcrop are primitive mountain brown forest soils. The area is experiencing a recreational load as it is situated next to a ski resort.

Biodiversity and dominance assessment

To determine the occurrence and the relative density of all species in each habitat, two transects were established, each with 25 quadrats (0.25 m^2). The distance between each quadrat was 1.5 m and between transects 3 and 4 m. There were 50 quadrats in each habitat. Species diversity, projective coverage (the percent of ground area occupied by the species), and the number of individuals of each species were determined in each quadrat. In addition to grass species, tree/shrub shoots up to 40 cm were also recorded.

The assessment of alpha diversity was carried out using the index of species richness—the number of species calculated per unit area (0.25 m^2).

Relative density (RD) is an estimate of the numerical strength of a species in relation to all other species, calculated as:

$$\mathrm{RD}\ (\%) = \left(\frac{\mathrm{N}_i}{\mathrm{N}_{all}}\right) \times 100, \tag{1}$$

where N_i is the number of individuals of a species; N_{all} the number of individuals of all species.

Frequency (F) is the distribution or dispersion of an individual species, estimated as percentage of occurrence:

$$\mathrm{F}\ (\%) = \left(\frac{\mathrm{Nq}_i}{\mathrm{N}_{qt}}\right) \times 100, \tag{2}$$

where Nq_i is the number of quadrates in which the species occurred; Nq_t the total number of quadrates.

Similarity in species composition between communities on serpentine and granite outcrops was calculated by

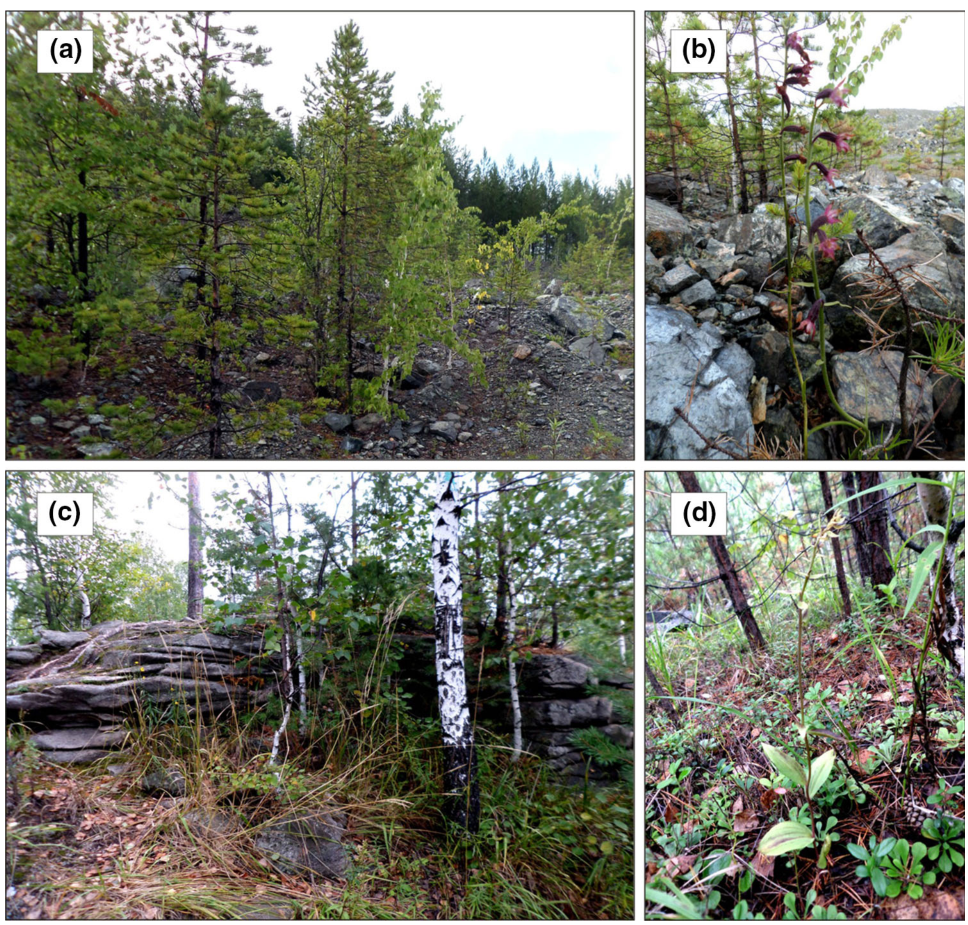

Fig. 2 Distant and close view of *E. atrorubens* growing on **a**, **b** serpentine and **c**, **d** granite outcrops

Sorenson's coefficient of community (Ks) (Smith and Smith 2012):

$$\mathrm{Ks} = \frac{2\mathrm{C}}{(\mathrm{S}_1 + \mathrm{S}_2)}, \tag{3}$$

where C is number of common species in both habitats; S_1 the number of species on the serpentine site; S_2 the number of species on the granite site.

An analysis of community heterogeneity was carried out with Simpson's Index for Dominance (D), calculated by the formula (Magurran 1988):

$$\mathrm{D} = \sum \frac{[\mathrm{n}_i(\mathrm{n}_i - 1)]}{[\mathrm{N}(\mathrm{N} - 1)]}, \tag{4}$$

where n_i is the number of individuals of each species; N the total number of individuals

To quantify species biodiversity, the Shannon–Wiener Diversity Index (H) was calculated (Mukhopadhyay et al. 2017):

$$\mathrm{H} = -\sum \mathrm{p}_i \ln(\mathrm{p}_i), \tag{5}$$

where p_i is the ratio between the number of individuals of each species to the total number of individuals (n_i/N).

Pielou eveness index (E), an indicator of community equitability, was calculated based on the Shannon–Wiener index:

$$\mathrm{E} = \frac{\mathrm{H}}{\ln \mathrm{S}}, \tag{6}$$

where H is Shannon–Wiener diversity index; S the total number of species; and ln the natural logarithm.

Sample collection and preparation

A 2000 m^2 (100 × 20 m) area was selected on both sites with an area along the slope. Soil and plant samples were collected in August 2017 and 2018. Five 2 kg samples were collected from the 15-cm soil layer using a transect (Maiti 2013) and transferred for physicochemical analyses (granulometric, pH, available nitrogen, phosphorus, total and available metal concentrations). All samples were mixed thoroughly, air-dried for five days, oven-dried at 45 °C for 24 h and sieved. Due to legal restrictions, only four generative plant specimens ($\sim$ 40 to 50 cm length) were collected from each site. Each plant was excavated with soil to preserve the roots, placed in sterile bags, and delivered to the laboratory. The material was cut into shoot and root parts, washed by ultrasonication UM-4 (Unitra Unima, Poland), and finally with deionized water. After measuring the number and length of adventitious roots, 1–2-cm roots were used to study mycorrhiza parameters; the rest of the materials was used to determine nitrogen, phosphorus and metal concentrations. Before chemical analysis, soil and plant samples were dried for 4 h at 75 °C.

Physicochemical characteristics of serpentine and granite substrates

The first soil portion was used to determine percentage of different grain sizes and was carried out by standard sieve analysis (stones: > 10 mm; gravel large: 10–5 mm; gravel small: 5–3 mm; sand large: 3–1 mm; sand average: 1–0.25 mm; dust and clay: < 0.25 mm). The second portion was cleaned to separate stones, sieved through a < 2 mm mesh and the weight reduced to 1 kg by the coning-quartering method (Maiti 2013) and used to determine pH, nitrogen, phosphorus, and metal contents. The pH of soil–water suspension (1:2.5; w/v) was measured using Anion 4100 pH-meter (NPP Infraspak-Analit, Russia). The potentially available nitrogen content was determined according to the method of Cornfield (Prasad 1965). Available forms of phosphorus were extracted in a 0.2 N water solution of HCl and measured using UV–visible spectrophotometer Specord-40 (Analytik Jena AG, Germany) (Bekuzarova et al. 2016).

Total nitrogen and phosphorus contents

Total nitrogen and phosphorus contents in *E. atrorubens* shoots and roots were measured spectrophotometrically at 440 and 640 nm, respectively, after wet digestion of material with an acid mixture of $HClO_4$ and H_2SO_4 (1:10; v/v). Total nitrogen was determined using Nessler's reagent, whereas total phosphorus was determined by the standard ammonium molybdate in acid medium as described earlier (Borisova et al. 2014).

Metal analysis

Total metal concentrations in soil, shoots and roots were determined after wet digesting with concentrated analytical grade nitric acid using MARS 5 Digestion Microwave System (CEM Corporation, US). The available forms of metals were analyzed by heating the 5 g soil sample with 10 mL of 0.5 M nitric acid in a water bath and stirring for 3 h at 90 °C in compliance with standardized methodology (M-MVI-80 2008). Samples were then filtered into a 50 mL flask and the filter washed with 40 mL of 0.5 M nitric acid. All samples were prepared using double deionized Millipore water (Milli-Q system, Millipore, France). Magnesium, calcium, potassium, iron, zinc, copper, manganese, nickel, chromium, lead, and cobalt concentrations were determined using a flame atomic absorption spectrometer AA240FS (Varian Australia Pty Ltd, Australia). Standard reference materials (JSC Ural

Chemical Reagents Plant, Russia) were used for the preparation and calibration of each analytical batch. Calibration coefficients were maintained at a high level of not less than 0.99. The bioconcentration factor (BCF) was calculated as the ratio of metal concentrations in shoots/-roots to its available concentration in the soil. The translocation factor (TF) was calculated as the ratio of metal concentration in the shoots to its content in the roots.

Biometric assessment of *E. atrorubens* and mycorrhizal colonization

Fifteen flowering *E. atrorubens* plants growing on serpentine and granite outcrops were used to study shoot lengths, inflorescence, number of leaves, flowers and fruits, and total leaf area under in situ conditions.

Previously prepared roots of *E. atrorubens* were used for studying mycorrhizal association. Root tips up to 1.5 cm were cross sectioned to 20 µm with a freezing microtome MEP-01 (Technom, Russia). Root sections of 50 samples from each habitat were analyzed under a light microscope Meiji MT 4300L (Meiji Techno, Japan) at 100-*x* magnification. The presence of pelotons or intercellular hyphal coils in the root sections was determined and the proportion of sections with pelotons calculated. This parameter reflects the uniformity of distribution of the fungal symbiont in the plant root or the frequency of occurrence of the fungus.

Statistical analysis

Statistical analyses were carried out to determine mean values and standard errors (SE) of the data. Differences between physicochemical and morphological parameters of the two sites were determined with the non-parametric Mann–Whitney *U*-test. Different letters in tables and figures indicate significant differences ($p < 0.05$) between the two sites.

Results and discussion

Biodiversity, dominance, and phytocoenotic analysis

The serpentine site was up to 35–40% rocky with predominant trees species being *Pinus sylvestris* L., *Betula pendula* Roth, *Populus tremula* L., *and Larix sibirica* Ledeb., growing in small groups or solitary. *Pinus sylvestris* was 20–25 years-of-age and 6–8 m in height. A young *Salix caprea* L., *S. cinerea* L., *Populus balsamifera* L., *P. suaveolens* Fisch. community (1.5–2.0 height) was present at the edge of the site. Vegetation was sparse with 3–5% grasses. Plants such as *Chamaecytisus ruthenicus* (Fisch. ex Wołoszcz.) Klásková, *Orthilia secunda* (L.) House, *Solidago virgaurea* L. grew under the trees. Petrophytic species, plants that grow on rocky outcrops, such as *Dendranthema zawadskii* (Herbich) Tzvel., *Dianthus versicolor* Fisch. ex Link, *Thymus talijevii* Klok. Et Shost., *Veronica spicata* L., *Rumex thyrsiflorus* Fingerh., *Euphrasia pectinate* Ten. and ruderal species, ones first to colonize disturbed lands, such as *Tussilago farfara* L., *Hordeum jubatum* L., *Melilotus albus* Medik., were found in open areas.

On the second site, *E. atrorubens* was growing on the flat top of Motaikha Mount with outcrops of large blocks of granite, up to 15% of the surface. The top of the mountain was covered with *P. sylvestris*. Mature trees were partially cleared and in their place, a young (up to 15-years-old, 5–7 m high), mixed stand of *P. sylvestris*, *B. pendula*, *L. sibirica* had formed. The canopy density of the trees reached 60%. Plants such as *Sorbus aucuparia* L., *Salix myrsinifolia* Salisb., *S. cinerea*, *Ch. ruthenicus* formed the undergrowth. The total projective cover of grass and shrub layers at the second site varied from 10% to 80%. The predominant species were *Calamagrostis arundinacea* (L.) Roth, *Vaccinium vitis-idaea* L., *Antennaria dioica* (L.) Gaertn., *Hieracium umbellatum* L., *Rubus saxatilis* L., *Polygonatum odoratum* (Mill.) Druce. In addition, there was extensive moss-lichen cover.

A more detailed analysis of vegetation biodiversity was carried out on the species found on the site transects. A total of 22 species was identified on the serpentine outcrop and 34—on granite (Table S1). Only 12 species were common to both sites (Table 1). Sorenson's coefficient of community (Ks) for the two *E. atrorubens* habitats was 0.4.

The distribution of species over the area was uniform for both sites. However, the *E. atrorubens* population P-1 (n = 305), was noticeably higher than P-2 (n = 36). The plant communities have equal Simpson's indices for dominance (D = 0.3), which can be explained by the same number of dominant species with higher abundance. The low level of the dominance index underlines the importance of a small number of species in the structure of plant communities.

The greatest relative density and frequency on serpentine outcrops were noted for *P. sylvestris*, *D. zawadskii*, *O. secunda* and *E. atrorubens*, whereas on granites these were noted for *A. dioica*, *V. vitis-idaea*, *C. arundinacea* and *P. sylvestris* (Table 1, Table S1).

The quantitative assessment of biodiversity using the Shannon–Wiener Index (H) revealed a difference in plant communities on both sites. On serpentine outcrops, H = 2.5 was found compared to H = 3.0 for the granite site. The degrees of uniformity of E = 0.8 in the distribution of the participation of species were the same on both sites. The higher Shannon–Wiener indices of granite

Table 1 Relative density (RD) and frequency (F) of species growing on both the serpentine and granite outcrops

Plant species	Family	Serpentine outcrops		Granite outcrops	
		Relative density (%)	Frequency (%)	Relative density (%)	Frequency (%)
Betula pendula Roth	Betulaceae	1.0	8.0	0.6	8.0
Calamagrostis epigeios (L.) Roth	Poaceae	0.6	6.0	0.6	8.0
Chamaecytisus ruthenicus (Fisch. ex Wołoszcz.) Klásková	Fabaceae	1.0	8.0	0.5	10.0
Epipactis atrorubens (Hoffm. ex Bernh.) Bess.	Orchidaceae	8.4	54.0	0.8	20.0
Hieracium umbellatum L.	Asteraceae	0.2	2.0	1.0	14.0
Orthilia secunda (L.) House	Ericaceae	9.4	12.0	1.5	10.0
Pinus sylvestris L.	Pinaceae	38.9	68.0	3.7	36.0
Poa trivialis L.	Poaceae	1.0	10.0	0.1	2.0
Silene nutans L.	Caryophyllaceae	0.2	2.0	0.2	6.0
Solidago virgaurea L.	Asteraceae	0.6	6.0	0.3	6.0
Veronica spicata L.	Plantaginaceae	1.2	12.0	0.2	4.0
Viola arenaria DC.	Violaceae	0.2	2.0	1.0	4.0

outcrops further indicates less favorable conditions for the growth of *E. atrorubens.*

This research has shown that plant communities are characterized by the dominance of a relatively few species. The P-1 population of *E. atrorubens* on the serpentine deposits has a higher size and density compared with the P-2. It is well known that orchids growing in secondary habitats are often abundant due to limited competition from other plants (Adamowski 2006). This research has shown that orchid population P-2 experienced phytocoenotic stress in which seed reproduction is difficult. This is confirmed by the increased Shannon–Wiener index indicating less favorable conditions for *E. atrorubens* on granite outcrops because of a competitive environment with other species.

Physicochemical characteristics of soils

Based on granulometric composition, serpentine substrates are mainly stony with 54–84% stones and gravel with diameters > 3.0 mm (Table 2). The rhizospheric soil zone of the granite massif was mainly sandy with sizes 0.25–3.0 mm, and the dust and clay fractions were 36%. The majority of the serpentine soil was dominated by skeletal part with sizes > 1.0 mm.

The pH of the serpentine soil was circumneutral (6.5–7.5) and the granite soil was weakly acidic. *E. atrorubens* prefers neutral or alkaline soils and avoids acidic soils. According to Vakhrameeva et al. (2008), the species mainly occurs in a pH range 7.5–9.0. However, populations of *E. atrorubens* in this study were growing both on a circumneutral (P-1) and on a weakly acidic substrate (P-2).

Table 2 Granulometric composition, pH and available nutrient contents of serpentine and granite soils

Parameters	Serpentine soil	Granite soil
Granulometric composition (%)		
Stones > 10 mm	33.3 *a*	1.4 *b*
Gravel large 10–5 mm	17.9 *a*	1.5 *b*
Gravel small 5–3 mm	14.2 *a*	2.7 *b*
Sand large 3–1 mm	17.4 *b*	22.9 *a*
Sand average 1–0.25 mm	11.1 *b*	35.5 *a*
Dust and clay < 0.25 mm	6.1 *b*	36.0 *a*
pH and available nutrients		
pH (soil–H_2O; 1:2.5; v/v)	7.2 ± 0.4 *a*	5.2 ± 0.2 *b*
Nitrogen (mg N kg^{-1} DW)	28.3 ± 2.3 *b*	59.0 ± 4.0 *a*
Phosphorus (mg P kg^{-1} DW)	22.0 ± 2.1 *b*	71.3 ± 5.2 *a*

Data presented mean values ± SE (n = 5)

SE standard error, *DW* dry weight

Different letters indicate significant differences between two sites according to Mann–Whitney *U*-test ($p < 0.05$)

The serpentine soil had low available nitrogen and phosphorus levels, whereas in the granite soil, their concentrations were higher on average by 2.5 times (Table 2).

Therefore, the present study showed that a serpentine substrate differed from a granite one by greater stoniness and a circumneutral pH. Such conditions obviously better favored *E. atrorubens* growth on a serpentine habitat and contributed to an increase in P-1 population size.

Total nitrogen and phosphorus content in *E. atrorubens*

The levels of total nitrogen and phosphorus in *E. atrorubens* shoots were significantly higher (by 1.2 and 1.3 times, respectively) on granite substrates compared with plants from serpentine outcrops (Fig. 3). Based on nitrogen content in the roots, there were no significant differences between the populations, while phosphorus in plants on the serpentine substrate was 1.3 times lower than plants on granite one.

The conditions of mineral nutrition are of special importance for the development of the photosynthetic apparatus. Nitrogen is a part of all proteins, chlorophyll, nucleotides, amino acids, amides, and alkaloids; therefore, it is one of the principal elements of living organisms, whereas phosphorus is also important as it is a constituent of biomembranes and macromolecular structures and plays an important role in cell metabolism (Marschner 1995).

The lower content of nitrogen and phosphorus in *E. atrorubens* shoots and roots on serpentine outcrops may be explained by their lower concentration compared to levels on granite. However, some reports indicate the ability of this species to grow on infertile substrates (Landolt 1977; Ellenberg et al. 1991; Vakhrameeva et al. 2008). Under nutrient deficient conditions, mycorrhiza promote their mobilization and uptake by plant roots (Smith and Read 2008; Vakhrameeva et al. 2008). This is confirmed by higher phosphate contents in the roots (almost 2 times) compared with the shoots.

Metal content of serpentine and granite soils and *E. atrorubens*

A high concentration of total Mg (205 g kg^{-1}) was found in the serpentine soil, some 79 times greater than in granite soil (Table 3). However, the difference in available Mg between the sites was less significant (23 times). The concentration of Mg in roots and shoots from the serpentine site was 8 times and 2 times higher than plants growing on the granite, respectively. Total and available Ca in the serpentine substrate was higher than in the granite (4 and 15 times, respectively). At the same time, levels in roots and shoots of *E. atrorubens* colonizing serpentine outcrops were slightly lower.

The difference between potassium concentrations among the two substrates was less noticeable and consecutively its content in the shoots was also the same (Table 3).

The bioconcentration factor (BCF) was in the order of $K > Ca > Mg$ in both habitats. The BCF for potassium was similar in both sites (Table S2).

The ratio of total Mg:Ca in the serpentine substrate was about 15, whereas it was 1 in the granite substrate (Table 3). However, the ratio of their available forms on both the sites was close to one. Magnesium level in roots from the serpentine site was 2 times higher than calcium concentrations, while the reverse was found for shoots (Table 3). Average Ca concentration in roots and shoots from the granite habitat was 5 times higher than Mg.

Potassium and calcium levels in the shoots were higher than in roots (Table 3). This was confirmed by the translocation calculation, which was above one for the both habitats. At the same time, the plants differed significantly in magnesium translocation, which was two for the granite

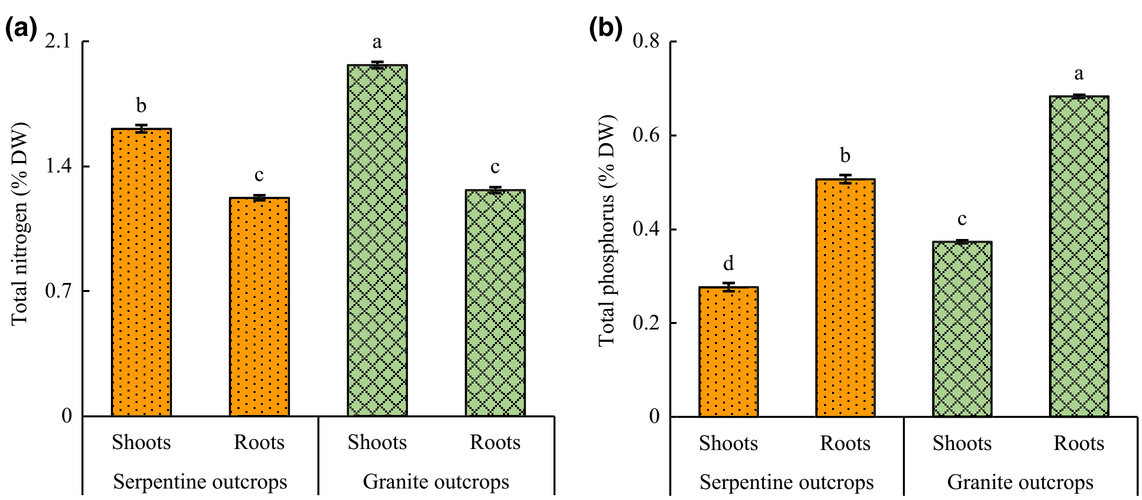

Fig. 3 Total nitrogen (**a**) and phosphorus (**b**) content in shoots and roots of *E. atrorubens* growing on serpentine and granite outcrops. Data presented: mean values ± standard error (n = 5). Different letters indicate significant differences between the two sites according to Mann–Whitney *U*-test ($p < 0.05$)

Table 3 Metal content in soil (total and available), roots and shoots of *E. atrorubens* growing on serpentine and granite outcrops

Metal, mg kg^{-1} DW	Mg (× 10^3)	Ca (× 10^3)	K (× 10^3)	Fe (× 10^3)	Zn	Cu	Mn	Ni	Cr	Pb	Co
Serpentine outcrops											
Soil (total)	205.4 ± 6.3 *a*	14.1 ± 0.4 *a*	4.1 ± 0.1 *a*	46.6 ± 0.9 *a*	30.5 ± 2.4 *b*	120.0 ± 3.7 *b*	794.2 ± 10.8 *a*	1617.7 ± 39.5 *a*	1263.3 ± 70.9 *a*	6.0 ± 0.5 *b*	60.6 ± 2.3 *a*
Soil (available)	9.0 ± 0.3 *a*	9.2 ± 0.2 *a*	0.2 ± 0.02 *a*	3.1 ± 0.1 *a*	1.8 ± 0.1 *b*	2.3 ± 0.1 *b*	205.0 ± 3.2 *b*	127.7 ± 3.0 *a*	1.7 ± 0.1 *a*	1.5 ± 0.04 *b*	6.2 ± 0.3 *a*
Roots	16.4 ± 0.4 *a*	9.3 ± 0.3 *b*	11.5 ± 0.8 *b*	1.1 ± 0.04 *a*	81.3 ± 5.3 *b*	14.5 ± 0.6 *b*	39.9 ± 2.7 *b*	86.0 ± 5.7 *a*	34.4 ± 2.0 *a*	3.1 ± 0.4 *b*	bdl
Shoots	11.4 ± 0.5 *a*	14.4 ± 0.3 *b*	21.3 ± 0.5 *a*	0.9 ± 0.01 *a*	78.8 ± 5.7 *b*	11.9 ± 1.0 *a*	30.4 ± 2.3 *b*	22.6 ± 1.7 *a*	23.8 ± 1.7 *a*	5.0 ± 0.5 *a*	bdl
Granite outcrops											
Soil (total)	2.6 ± 0.1 *b*	3.6 ± 0.1 *b*	1.9 ± 0.1 *b*	12.8 ± 0.3 *b*	202.3 ± 6.2 *a*	260.7 ± 7.0 *a*	459.3 ± 8.8 *b*	17.3 ± 1.1 *b*	21.3 ± 1.6 *b*	67.0 ± 2.0 *a*	3.5 ± 0.2 *b*
Soil (available)	0.4 ± 0.02 *b*	0.6 ± 0.02 *b*	0.2 ± 0.01 *a*	2.2 ± 0.05 *b*	119.0 ± 2.6 *a*	63.7 ± 1.4 *a*	351.2 ± 5.8 *a*	3.7 ± 0.1 *b*	1.5 ± 0.02 *b*	50.0 ± 1.6 *a*	0.8 ± 0.1 *b*
Roots	2.1 ± 0.1 *b*	11.6 ± 0.4 *a*	16.1 ± 0.8 *a*	1.2 ± 0.07 *a*	1110.5 ± 55.7 *a*	65.4 ± 1.7 *a*	126.7 ± 7.2 *a*	18.4 ± 1.7 *b*	8.6 ± 0.8 *b*	19.4 ± 1.1 *a*	bdl
Shoots	4.7 ± 0.2 *b*	18.6 ± 0.9 *a*	22.1 ± 0.5 *a*	0.4 ± 0.03 *b*	204.5 ± 4.6 *a*	19.7 ± 1.2 *b*	41.6 ± 2.6 *a*	12.2 ± 0.9 *b*	7.8 ± 0.7 *b*	5.6 ± 0.5 *a*	bdl

Data presented mean values ± SE (n = 5)

SE standard error, *DW* dry weight, *bdl* below detection limit

Different letters indicate significant differences between the two sites according to Mann–Whitney *U*-test ($p < 0.05$)

substrate and less than one for the serpentine substrate (Table S2).

High concentrations of total nickel, chromium, cobalt and iron were also found in the serpentine soil; the Ni content was 94 times, Cr—59 times, Co—17 times and Fe—4 times higher than in the granite soils. Nevertheless, available nickel in serpentine soils was 35 times and cobalt 8 times higher than in the granite soils, whereas iron and chromium level differences between sites averaged 20%. Differences between the sites on metal contents in roots and shoots were minor; average contents of nickel and chromium in plants on serpentine soil was about 4 times higher than plants on granite soil. Total and available lead, zinc and copper in granite soil was considerably higher than in the serpentine soil (Table 3).

The bioconcentration factors for shoots and roots of *E. atrorubens* colonizing a serpentine habitat were in the order of Zn > Cr > Cu > Pb > Ni (Fe) > Mn (Table S2). The BCF for the first four metals were greater than one, while the BCF for *E. atrorubens* on a granite soil were highest for Zn, Ni and Cr.

Levels of the metals in *E. atrorubens* roots were higher than in the shoots (Table 3). The metal translocation factor was below one (Table S2). The translocation factor for *E. atrorubens* colonizing the serpentine substrate was: Pb > Zn > Cu > Fe > Mn > Cr > Ni, while on the granite substrate it was Cr > Ni > Fe ≥ Mn > Pb > Zn > Cu.

Plants which are able to grow on serpentine soils show specific morphological and physiological adaptations, called “Syndrome” (Kazakou et al. 2008, 2010). Previously, it had also been reported that *E. atrorubens* grew luxuriantly on serpentine sites in Serbia without any detrimental effects (Djordjević et al. 2016).

This study shows that a possible reason for increased *E. atrorubens* tolerance to high metal concentrations in the serpentine soil is its ability to accumulate more metal in the root system and prevent its transfer to aboveground organs, suggesting its excluder strategy.

Biometric assessment of *E. atrorubens* and mycorrhizal association

Morphological analysis of two *E. atrorubens* populations showed no significant differences for shoot length and number of flowers per inflorescence between both sites (Table 4). This was found previously for leaf area (Filimonova et al. 2019). Leaves of plants growing on serpentine outcrops, as shown by this study, were significantly (about 2 times) thicker compared to plants growing on the granite substrate. This was due to an increase in the number of mesophyll cell layers and epidermal thickness in P-1 plants. The formation of a thicker leaf blade was the result of a higher light intensity and radiation in an open habitat.

Table 4 Morphological characteristics of *E. atrorubens* on serpentine and granite outcrops

Site	Shoot length (cm)	Inflorescence length (cm)	Number of flowers per inflorescence	Number of fruits per plant	Number of leaves per plant
Serpentine outcrops					
Mean ± SE	48.7 ± 5.0 *a*	14.7 ± 2.1 *a*	17.8 ± 3.4 *a*	13.4 ± 3.9 *a*	7.9 ± 0.3 *a*
Range	35.5–64.8	7.9–24.7	10–27	5–25	7–9
Granite outcrops					
Mean ± SE	46.1 ± 2.3 *a*	8.7 ± 1.3 *b*	11.0 ± 2.3 *a*	2.7 ± 1.4 *b*	6.8 ± 0.3 *b*
Range	40.8–55.0	5.0–12.6	6–19	0–8	6–8

Different letters indicate significant differences between the two sites according to Mann–Whitney *U*-test (n = 15, $p < 0.05$)

E. atrorubens is a light-demanding species (Vakhrameeva et al. 2008; Djordjević et al. 2016). An increase in leaf thickness may contribute to more efficient absorption and use of light energy in high insolation conditions. In addition, it could also compensate for the lack of photosynthetic pigments found previously (Filimonova et al. 2019).

The proportion of fruit formation was 74% on the serpentine outcrops, whereas it was significantly reduced (up to 11%) on the granite substrate (Table 4).

P-1 plants formed a better developed root system in comparison with plants on P-2. Average root numbers on the serpentine site was 59, with lengths up to 540 cm, whereas plants on granite outcrops averaged 24 roots with lengths of 180 cm.

Orchid mycorrhizae, represented by pelotons, were found in the root cells of *E. atrorubens* from both sites. In the early stages of development, *E. atrorubens* is an obligate mycosymbiotroph, whereas in the adult stage, the intensity of mycorrhizal association is mild to moderate and slightly depends on the fungi (Vakhrameeva et al. 2008; Tešitelová et al. 2012). *E. atrorubens* plants on granite outcrops had 82% pelotons (per unit root length), while it was only 50% on serpentine soil. The lesser numbers of root pelotons on serpentine soils may be associated with a lesser degree of plant community formation as well as with a high stony substrate which prevents the even distribution of the fungal mycelium. A smaller number of pelotons per unit root length of plants on serpentine substrate was compensated by longer root length.

Jurkiewicz et al. (2001) reported that mycorrhizae play an important role in the detoxification of metals in *E. atrorubens* roots and therefore, increase the resistance of the species to high metal concentrations in the environment. They have shown that heavy metals are stored in cell walls of fungi. This study shows an accumulation of most of the metals in the roots was significantly higher than in the shoots for both sites. It is possible that mycorrhizae make the *E. atrorubens* tolerant to high concentrations of heavy metals in serpentine soils.

On rich soils, *E. atrorubens* is strongly affected by more competitive species, which results in a decrease in population, seed productivity, and capacity to compete with other plant species (Vakhrameeva et al. 2008). In our studies, this was also evident by the less developed root system of individuals on granite substrate compared with plants on serpentine outcrops.

Conclusions

This research has revealed the main differences between the two forest plant communities. The degree of biodiversity, relative density and frequency of most of the species as well as the density of canopy and projective coverage of the grasses were higher on the granite site compared with the serpentine one. Therefore, the population of *E. atrorubens* growing on granite outcrops experiences phytocoenotic stress, in which seed reproduction is difficult. The population was noticeably lower. *E. atrorubens* colonizing the serpentine deposits were characterized by the formation of larger number of fruits, greater root lengths, and thicker leaf blades. They showed high viability despite lower nitrogen and phosphorus levels as well as higher metal concentrations compared to plants on the granite outcrop. The levels of most of the metals were considerably higher in the roots than in the shoots. The well-developed orchid mycorrhizae also obviously contributed to the survival of this species. Hence, serpentine outcrops formed due to the mining of asbestos could be a suitable substrate for the light-demanding *E. atrorubens* because of their ability to adapt to dry, rocky and nutrient-depleted soils and limited competition from other species. Future research will include identifying adaptive physiological mechanisms that contribute to the survival of orchids under extreme detrimental conditions of serpentine deposits.

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